Observation of Time-resolved Collapse of a Folding Protein

High-intensity, "pink" beam from an undulator was used in conjunction with microfabricated rapid fluid mixing devices to monitor the early events in protein folding with time-resolved small-angle x-ray scattering. This brief report describes recent work on the protein bovine β-lactoglobulin in which collapse from an expanded to a compact set of states was directly observed on a millisecond time scale.

The question of how a protein changes shape ("folds") from a quasilinear state to assume its native, folded form is of both fundamental and practical interest [1]. Ideally, a fully unfolded peptide chain (denatured protein) forms an expanded random coil. In contrast, a folded protein is compact, with a density approaching that of an amino acid crystal and with well-defined internal (secondary and tertiary) structures. Chain collapse and secondary structure formation are included in the critical initial steps of protein folding that convert the former states to the latter. In some cases, a non-native compact state (known as compact denatured/molten globule/intermediate [2]) is rapidly formed. Folding to the native state occurs on a longer time scale. The relevance of rapidly formed, compact denatured states, specifically an understanding of their role in protein folding, is a topic of current controversy and interest [3-5]. Chain collapse can occur rapidly, typically much faster than the millisecond dead times of conventional mixing apparatuses, requiring experimental studies of folding with submillisecond time resolution to probe these states. Access to this regime has been achieved only very recently, with the advent of new technologies [6,7].

Small-angle x-ray scattering (SAXS) can be used to assess both the size and compactness of a protein in solution [8]. Other experimental techniques, used previously, measure these parameters less directly. Several groups have been using SAXS

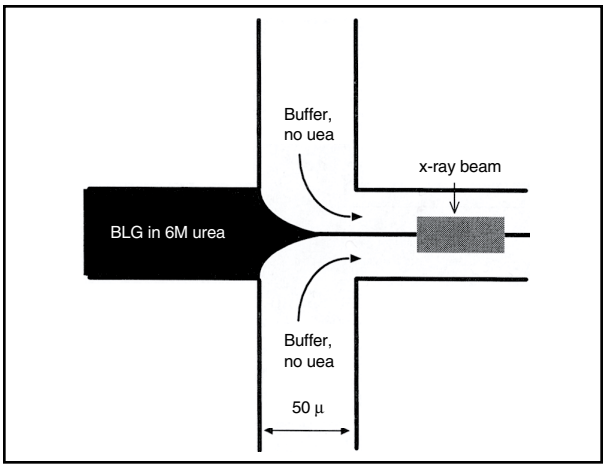

FIG. 1. A schematic of the rapid fluid mixer. In an experiment run, 1 mM unfolded protein in dilute phosphoric acid and 6 M urea (pH 2.8) flows in the inlet channel. Dilute phosphoric acid (pH 2.8) with no urea flows in the side channels, focusing the protein into a stream that is, on average, 5 µm wide. Small molecules, such as urea, diffuse rapidly out of the central stream, triggering the folding reaction. The pH was maintained below 3 at all times to prevent dimerization of the BLG (Ref. 13). (The BLG used for these experiments was expressed and purified from the yeast Pichia pastoris.*) The sample temperature was maintained at 27°C throughout the experiment. The device was fabricated as described in Ref. 9. For this work, the channel width has been reduced by a factor of 2, to 50 µm, while the channel depth remained 390 µm. The higher aspect ratio results in a more uniform stream as a function of distance from the top and bottom sealed surfaces, which, in turn, results in more uniform diffusion times. One possible position of the x-ray beam is shown.*

to study protein folding [9–13]. Time-resolved SAXS (TRSAXS), with millisecond or faster resolution, can provide the requisite information about transient states, including partially folded states [14]. This paper reports significant advances in TRSAXS experiments using microfabricated fluid mixers in conjunction with small, brilliant x-ray beams available at the Advanced Photon Source (APS). We have studied the initial stages of the folding of β-lactoglobulin (BLG), a 162 amino acid, β-barrel protein found in bovine milk. Very recently, rapid mixing experiments using both Trp fluorescence and hydrogen exchange labeling indicate the presence of many kinetic phases in BLG folding [15]. In these experiments, signatures that are consistent with collapse are observed within 2 ms of the initiation of folding. A schematic of the mixer is shown in Fig. 1.

The SAXS experiments were performed at IMM-CAT (APS) where pink beam (full width at half maximum = 2.6%) was produced by reflecting the first harmonic of APS undulator A (7.65 keV at a gap of 18 mm) off a Si mirror [16]. The beam was collimated with a pair of crossed slits located 14 cm upstream of the sample and was set to 10 μm in the vertical direction, to illuminate only the protein jet, and 40 μm in the horizontal direction, to achieve the requisite time resolution of 240 μs per data point (at our average flow speed of 16.6 cm/s). The flux through the slits was 2.5 x 10^{11} x-rays/s. Exposure times were 40 s; typically two or more images were averaged. Pink beam can be used only with a flowing sample because of the potential for radiation damage. Scattered x-rays were detected using a custom-built CCD detector [17] located 40 cm downstream of the sample.

Kratky plots (Iq^2 versus q) can be used to show the power-law dependence of the high-q scatter. A peak in this plot indicates that the scatter falls off more rapidly than q^{-2}, a result typical of a compact, uniform object. Expanded objects have scattering that falls off as q^{-1} [8], hence the product Iq^2 continues to increase over a broad range of q. Thus, the appearance of a peak in the Kratky plot is useful for assessing collapse of the molecule. The main

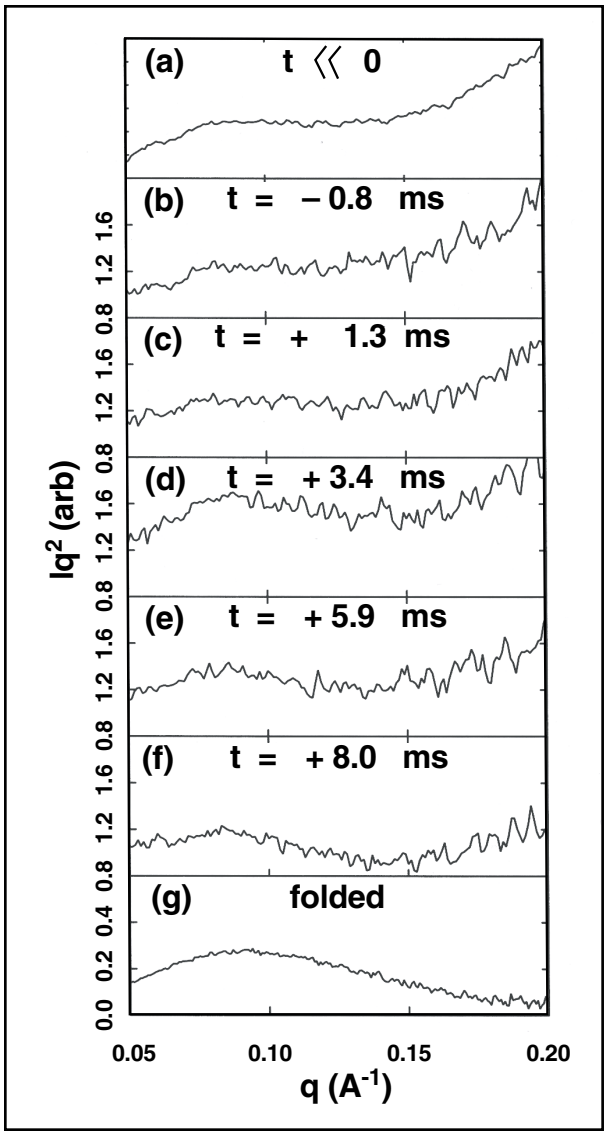

FIG. 2. Kratky plots showing collapse of the protein as a function of time, where t = 0 is defined to be the point at which the urea concentration is 3 M (native conditions). Collapse occurs between frames (c) and (d).

result of this work, Kratky plots of BLG flowing inside the device, is shown in Fig. 2. In this series of graphs, the product Iq^2 is plotted as a function of q for six different positions inside the device. The top frame shows protein in the inlet channel in the initial state (6 M urea, pH 2.8). The increase at high q indicates that this denatured state is expanded. The remaining frames show data taken at different locations along the outlet channel: (b) 0.8 ms before the urea concentration drops below 3 M (nearly identical to top trace, but with less S/N), (c) 1.3 ms after urea, 3 M, (d) 3.4 ms after, (e) 5.9 ms after, and (f)

8.0 ms after. A peak develops between frames (c) and (d), indicating some compaction on a time scale of 2 ms. By scaling the data of (b) to that of (e) and (f) in a range of q where we expect most of the contribution to come from the expanded state, we estimate that, by 6 ms, approximately 20% of the protein has become compact, and, by 8 ms, 30% of the protein has become compact. The high-q data taken at a second, higher protein concentration (2.5 mM, not shown) reproduce this trend. The last frame (g) shows data from a much larger (static) native sample of protein (data not acquired inside the mixer). In a control experiment, in which the solution surrounding the protein was the same as that flowing in the side channels, no change in the state of the protein was detected.

Experiments such as this one have the potential to distinguish between two very different folding mechanisms. In the first, a protein passes through a series of cooperative (all or nothing) transitions on its way to assuming its folded conformation. In the second, the protein passes through a series of partially folded states. One model of these states, discussed in Ref. 14, is of a compact core surrounded by random coil loops. The key to distinguishing between these cases lies in obtaining good statistics for the mid-q to high-q data.

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